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DEVELOPMENTAL BRAIN RESEARCH

Research Report

# Effects of insulin-like growth factors on organotypic cocultures of embryonic rat brainstem slices and skeletal muscle fibres

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**Abstract**

Embryonic rat brainstem slices including the facial and hypoglossal motor nuclei were maintained in organotypic cocultures with explants of embryonic tongue or post-natal skeletal muscle for periods up to 3 weeks. Survival and differentiation of motoneurones were dependant both on the type of muscle explant and its position relative to the brainstem. Tongue explants induced a more important glial outgrowth, a motoneurone migration towards the muscle, earlier muscular contractions and a more complete neuronal and muscular differentiation. Since the foetal tongue contains IGF levels as high as foetal liver, these effects might be due in part to diffusion of IGF from the explanted muscle. Indeed, foetal liver explants or crude foetal liver extracts induced effects similar to those of tongue explants. These effects can be reproduced by addition of IGF-1 or IGF-2, or both, into the culture medium. Although IGF-1 and IGF-2 had similar effects, IGF-1 induced a more pronounced muscular differentiation and IGF-2 promoted neuronal differentiation. Our results suggest that IGFs are good candidates as muscle-derived neurotrophic factors promoting survival and differentiation of rat cranial motoneurones. These results also stress the importance of neuroglial trophic interactions and target development.

*Key words:* Insulin-like growth factor; Muscle-derived neurotrophic factor; Facial nucleus; Hypoglossal nucleus; Cranial motoneuron; Sensory brainstem structure; Lumbar muscle; Tongue; Trophic effect

## 1. Introduction

The development of neuromuscular connections involves complex processes which are probably related to different types of trophic interactions. In vitro studies on dissociated motoneurones have clearly demonstrated the importance of target derived neurotrophic factors for the survival of motoneurones [41,42,43, 44,45]. Conversely, at the postsynaptic level, the presence of a motor innervation and the release of acetylcholine by nerve terminals are both necessary for the survival and differentiation of myotubes [13,25,57]. Organotypic neuromuscular cultures, in which a variety of cell types and interactions are maintained, may offer a good model to investigate the processes involved in the maturation of neuromuscular connections.

We have recently described an organotypic culture of embryonic rat brainstem slices in which cranial motoneurones survive, differentiate and innervate newly formed muscle fibres [22]. We have demonstrated that, in the absence of muscle explant, motoneurone pools disappeared within the first two weeks in culture, suggesting a high trophic effect in the presence of a muscle explant. This was characterized by a flattening of the brainstem and the development of a large monolayer region between the brainstem explant and the muscle tissue, into which the motoneurones migrate and differentiate. Although the original muscle tissue degenerates and new myotubes derive from dividing satellite cells, all muscle explants did not have the same trophic effect. Maturation and differentiation of cocultures were observed with differentiated muscle tissue from newborn animals but they were enhanced with tongue muscle explants from E18 embryos, leading to earlier contractions.

Foetal tongue contains high levels of IGF mRNA

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[3]. IGF is present in the brain [4,5,48,49,50] and its level of expression is correlated to development [55,56]. The present work was done to investigate the hypothesis that IGF promotes brainstem motoneuronal differentiation and the development of early neuromuscular connections in the organotypic slice culture. Part of this work has been published in abstract form [14].

## 2. Materials and methods

### 2.1. Preparation of brainstem explants

Cultures were done as previously described [22]. Briefly, brainstem explants were obtained from rat embryos on embryonic days 18 (E18) to 20 (E20). At this stage, motoneurones have migrated to their final locations and motor nuclei can be clearly identified on brainstem sections, but the neurones are not yet fully differentiated.

Pregnant Wistar female rats were anaesthetized with ether. After thoroughly cleaning the abdomen with 70° alcohol, the animal was decapitated. The two horns of the uterus, each containing 4 to 6 embryos, were removed and placed in a sterile Petri-dish. All further preparations were carried out under a laminar flow hood with sterile procedures. The wall of the uterus was opened and the intact amniotic sacs were freed and transferred to a new Petri dish containing Gey's balanced salt solution (GBSS) cooled to 15°C. The embryo was exposed by tearing open the sac, immediately decapitated and the head was removed to fresh cooled GBSS. Under a stereo microscope, the cranium was carefully opened along the mid-line and the brain gently lifted out with a small smooth spatula and transferred to a Petri-dish containing fresh cooled GBSS. Using a razor blade, a block of tissue containing the brainstem was isolated with two transverse sections made at the level of the caudal part of the cerebral hemispheres and the caudal medulla. The meninges and blood vessels were carefully peeled away with fine forceps under the stereo microscope and the clean brainstem was removed to fresh GBSS.

Brainstems were pooled and arranged on the base plate of a tissue chopper (McIllwain). Transverse sections, 275 $\mu$m thick, were obtained. Sections were collected with a moist spatula and dispersed in cool GBSS.

Sections containing oculomotor nucleus, motor trigeminal nucleus, facial and abducens nuclei or hypoglossal nucleus together with dorsal nucleus of the vagus nerve and nucleus ambiguus were identified under the stereo microscope and explants obtained by cutting away unwanted tissue. Sections were made in order to promote cellular and neuritic outgrowth [1] and to reduce the size of the explant. The final explants were removed to Petri dishes.

### 2.2. Preparation of muscle and liver explants

Tongues and livers were obtained from the same E18–E19 embryos and lumbar muscles from newborn (PN2-PN11) rats. Explants were obtained by chopping the muscles or the livers twice with 400 $\mu$m steps on a tissue chopper. After the first passage, the base plate was rotated 90° and a second passage was made to obtain small muscular fragments. Explants were collected and dispersed in fresh GBSS.

### 2.3. Embedding and coexplantation of brainstem tissue, muscle and liver

A brainstem explant and a small piece of muscle and, in some cases, of liver, selected under the stereo microscope were embedded together in a plasma clot on a perfectly cleaned glass coverslip according to the procedure previously described by Gähwiler [19]. Explants were placed in 40 $\mu$l of reconstituted chicken plasma (Sigma P3266) which was then coagulated by carefully mixing with 20 $\mu$l of Thrombine (Sigma T4648, 0.5 mg/ml). Before coagulation was complete, the different explants were precisely positioned.

### 2.4. Maintenance of cultures

The cultures were maintained according to the procedure described previously [6,22]. After coagulation of the plasma clot, the coverslips were introduced into conical vented plastic tubes containing 1.5 ml of culture medium. The tubes were placed on the drum of a tube roller, tilted 5° from the horizontal plane and set to 120 revolutions per hour, inside an incubator. Incubation takes place at 36°C in a dry atmosphere with an initial concentration of 5.5% $CO_2$. Carbon dioxide concentration changes were made according to Braschler et al. [6]. Medium changes (1.5 ml of fresh medium) were performed on days 5, 9, 12, and 14. Further changes were made every second day and medium replaced by 2.0 ml of fresh medium. Culture medium consisted of 48.6% of Dulbecco's modified Eagle's medium with 0.03% glutamine, 24.4% of Hank's BSS, 8.1% of heat inactivated foetal calf serum, 2.3% of 20% glucose solution and 16.6% of ultra-purified water. Osmolarity of the medium was measured and kept in the range 290–300 mOsm/kg $H_2O$. To promote the growth and differentiation of cholinergic cells [20,21], NGF was added to a final concentration of 20 ng/ml during the first week and 5 ng/ml thereafter. In some cases, 2 or 5% of crude liver extract or 10 ng/ml of human recombinant IGF-1 (Gibco 6203245SA) or IGF-2 (Gibco 6203255SA) were added to the medium at each medium change. Antibiotics were added to the first medium and suppressed in further medium changes.

### 2.5. Preparation of crude liver extract

Crude liver extracts were obtained from 2.4 g of fresh embryos livers collected in GBSS. Tissue was fragmented to small pieces with scissors and volume was extended to 10 ml with GBSS. The extract was homogenized by sonication and centrifugated for 30 mn at $48000 \times g$. The supernatent was collected and sterile filtered. It was added to the culture medium at concentrations of 2% or 5%.

### 2.6. Acetylcholinesterase staining

Cultures were fixed for 60 min in 4% formaldehyde and 0.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) and further processed as whole mounts for acetylcholinesterase (AChE). After washing in phosphate buffer, the cultures were incubated for 60 mn at 36°C in the medium of Karnovsky and Roots [30]. In order to increase penetration of the substrate, 3% Triton X-100 was added. Acetylcholine iodide was used as substrate and 4 mM tetraisopropyl pyrophosphamide (iso-OMPA) was added to inhibit non-specific esterases. The cultures were dehydrated in a graded series of ethanol and mounted upside-down under the original coverslip. In some cases, the cultures were lightly counterstained with Cresyl violet.

## 3. Results

The data base for this report stems from twelve experimental series. Each experimental series comprised 70 cultures including an average of 35 cultures of the facial region and 35 cultures of the hypoglossal region. Since results may vary widely from one experi-

ment to the next, each experimental series included a control and a test group for each region studied, and comparisons refer to these controls. Cultures with the same type of muscle explant as the test group, but positionned 1 mm ventrally and maintained in standard medium were used as controls. Each group in each series comprised a minimum of 10 cultures. Each trial was reproduced at least once in another experimental series. The trophic effects described in this work were observed with all motoneurone populations studied and were not seen on controls.

Embryonic brainstem explants containing cranial motor nuclei grown in organotypic cultures with explants of embryonic tongue muscle gave different results according to the distance and position of the muscle explant with regard to the nervous tissue. When the cultures were grown as described previously [22], with a muscle explant positioned 1 mm ventrally on the mid-line (control situation), the brainstem tissue spread symmetrically and the motor nuclei on each side survived, spread toward the monolayer region and stained strongly AChE-positive (Fig. 1A). Motoneurones differentiated and innervated striated muscle fibres with well defined motor end-plates. Contractions were observed as early as 6 days in vitro (DIV). The dorsal region containing sensory structures developed extensively in a direction opposite to the muscle explant.

If the muscle explant was positioned sideways, 1 mm on the left side of the brainstem explant (Fig. 1B), the cultures grew in a very different way. The proximal half of the brainstem spread in direction of the muscle explant. Motoneurones survived and differentiated, showing a well developed dendritic tree and the muscle displayed strong contractions. On the contrary, the distal half showed little spread and the motoneurone population tended to disappear. Dorsal spread of sensory structures was restricted to the proximal side (Fig. 1B).

The trophic effect of the muscle explant depended on the distance between the two explants. When the muscle explant was positioned on the mid-line, but 2 mm ventrally (Fig. 1C), the brainstem did not spread and the motor nuclei disappeared. This situation was similar to that observed in absence of a muscle explant [22].

These results show that the muscle explant exerted a strong trophic effect on the brainstem tissue possibly due to a diffusible substance.

When muscle explants were taken from lumbar muscles on newborn rats (PN2-PN11) (Fig. 2A), the spread of nervous tissue was less prominent and neuronal differentiation was reduced. Muscle tissue regenerated poorly and showed only numerous small, weakly AChE-positive myotubes, and few transversely striated muscle fibres. Contractions were not observed before 10 DIV.

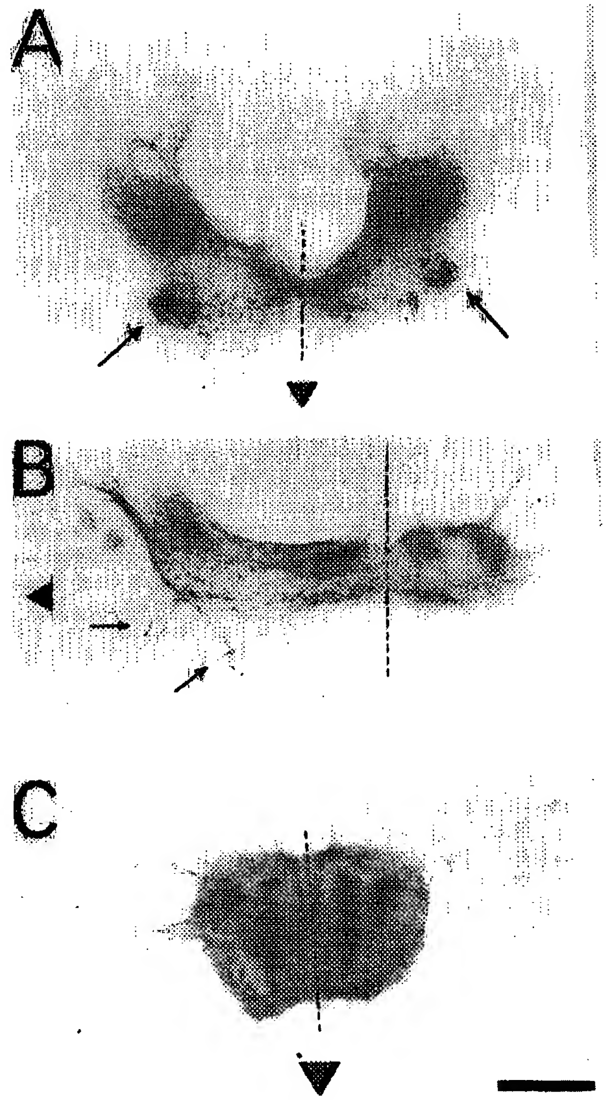



Fig. 1. Trophic effect of embryonic tongue explant on brainstem cultures at 13 DIV. Low power photomicrographs of brainstem explants of the facial nucleus region. AChE staining with Cresyl violet counterstaining. Dorso-ventral mid-line is indicated by doted line, dorsal region upwards. Large arrow heads point to the direction of the muscle explant. A: control culture with tongue explant positionned 1 mm ventrally on the mid-line. Abducens and facial motor nuclei are clearly seen (arrows point to the facial nuclei). Notice the important dorsal spread of the vestibular structures. B: tongue explant positionned 1 mm to the left. Notice the dissymetrical organization of the explant. Arrows point to migrated and differentiated motoneurones. C: tongue explant positioned 2 mm ventrally on the mid-line. Notice the lack of spread and the disappearance of AChE-positive motor nuclei. Bar = 1 mm.

Adding a small explant of liver tissue to lumbar muscle-brainstem cocultures had effects similar to those observed with tongue explants. The liver explant induced a clear outgrowth of brainstem tissue and AChE-positivity was better retained. A typical case is illustrated on Fig. 2B. An explant of liver was posi-

tioned sideways on the right of the brainstem whereas the lumbar muscle explant was located 1 mm ventrally. After 12 DIV, the culture showed a significant spread of the nervous tissue, especially in the direction of the liver explant, giving a clearly asymmetrical culture. The outgrowing tissue from liver and brainstem bridged the gap between the two explants. On the contrary, very little spread was seen in the direction of the muscle explant and the muscle and brainstem explants were not connected by outgrowth tissue. The original muscle fibres remained strongly AChE-positive and did not degenerate, but no newly formed muscular tissue was seen. In these conditions, no contractions were observed.

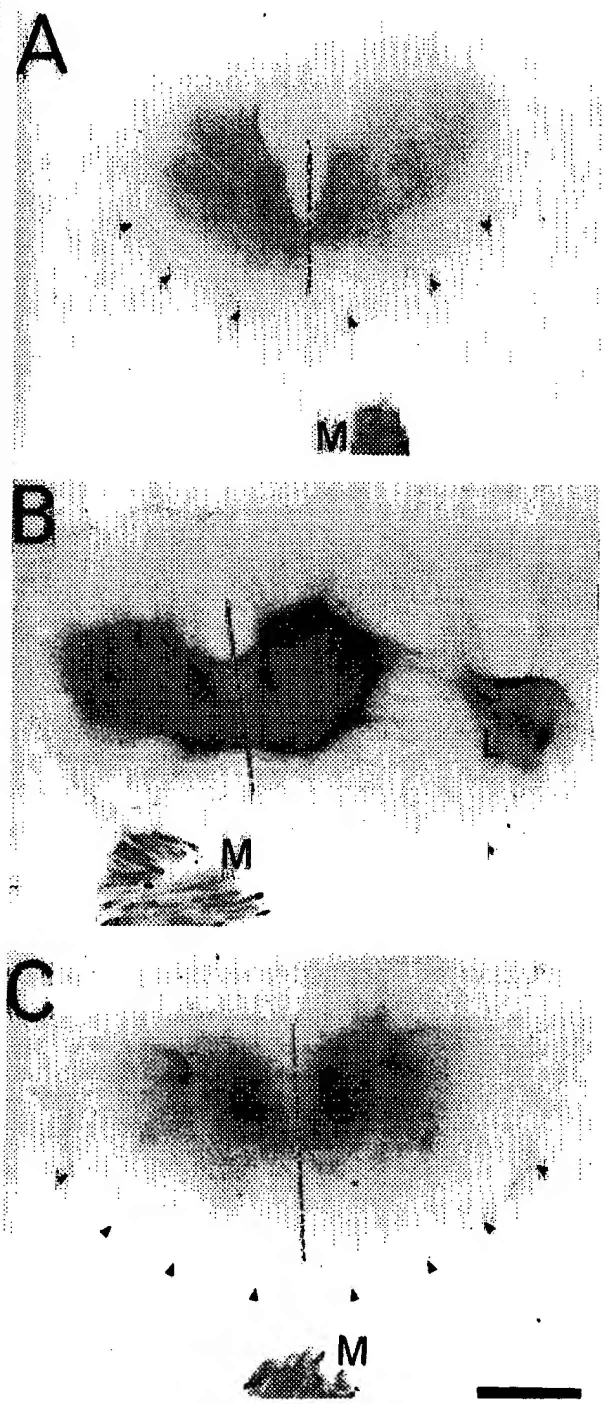


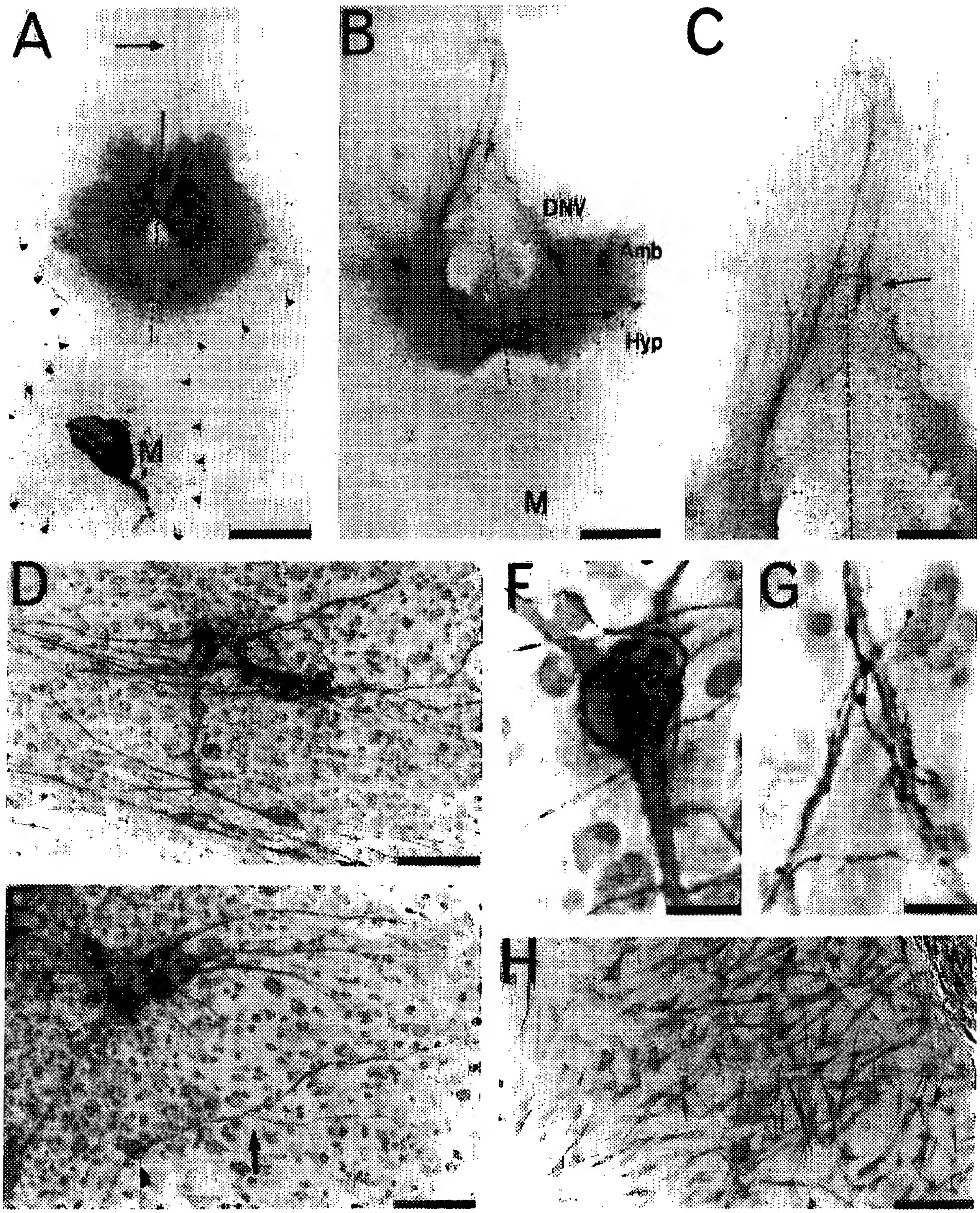

Fig. 2. Cultures of embryonic brainstem slices with lumbar muscle explants, at 8 DIV. Low power photomicrographs of brainstem explants of the facial nucleus region. AChE staining with Cresyl violet counterstaining. Dorso-ventral mid-line is indicated by doted line, dorsal region upwards. Arrow heads delineate tissular outgrowth. M: original muscle explant; L: foetal liver explant. A: control culture with lumbar muscle explant from an 11 day old pup. B: same situation as A, but with a foetal (E18) liver explant added 1 mm to the right of the brainstem. Notice outgrowth towards the liver explant. C: same situation as A, but with 2% of crude foetal liver extract added to the culture medium. Increased tissular spread and AChE-positive motoneurones and dorsal structures can be observed. Bar = 1 mm.

We next tried to replace the liver explant by embryonic liver extract added to the culture medium at concentrations of 2 or 5%. With 2% foetal rat liver extract, we observed an even spread of the brainstem tissue in all directions (Fig. 2C) after 1 week in culture. Survival of motoneurones pools was improved. Groups of motoneurones stained AChE-positive and some cells migrated slightly out of the brainstem. Little effects were seen on lumbar muscle fibres which regenerated poorly.

With 5% crude liver extract, motor nuclei survived well and stained strongly AChE-positive. After 20 DIV, they contained well differentiated motoneurones (Fig. 3E,F,G), with long AChE-positive dendrites covered with numerous spines. Afferent axons were seen running along each dendrite and apparently connecting the spines (Fig. 3F,G). Prominent trophic effects were also observed in the dorsal structures, especially in the dorsal nucleus of the vagus nerve and the nucleus of the solitary tract where some neurones stained strongly positive for AChE and sent long neuritic processes dorsaly into the plasma clot (Fig. 3B,C,D). Some outgrowth was also observed around the muscle explant (Fig. 3A,H).

Embryonic tongue and liver tissues are known to hybridize strongly for IGF mRNA and liver is the major source of IGF. This led us to consider that the effects induced in presence of tongue or liver in our preparation might be due to IGFs effects on brainstem and muscle tissue. To check this point, cultures were performed in medium supplemented with 10 ng/ml of human recombinant IGF-1 or IGF-2.

After 12 DIV, both IGF-1 and IGF-2 induced a significant spread of brainstem tissue, flattening of the cultures, survival and differentiation of motoneurone

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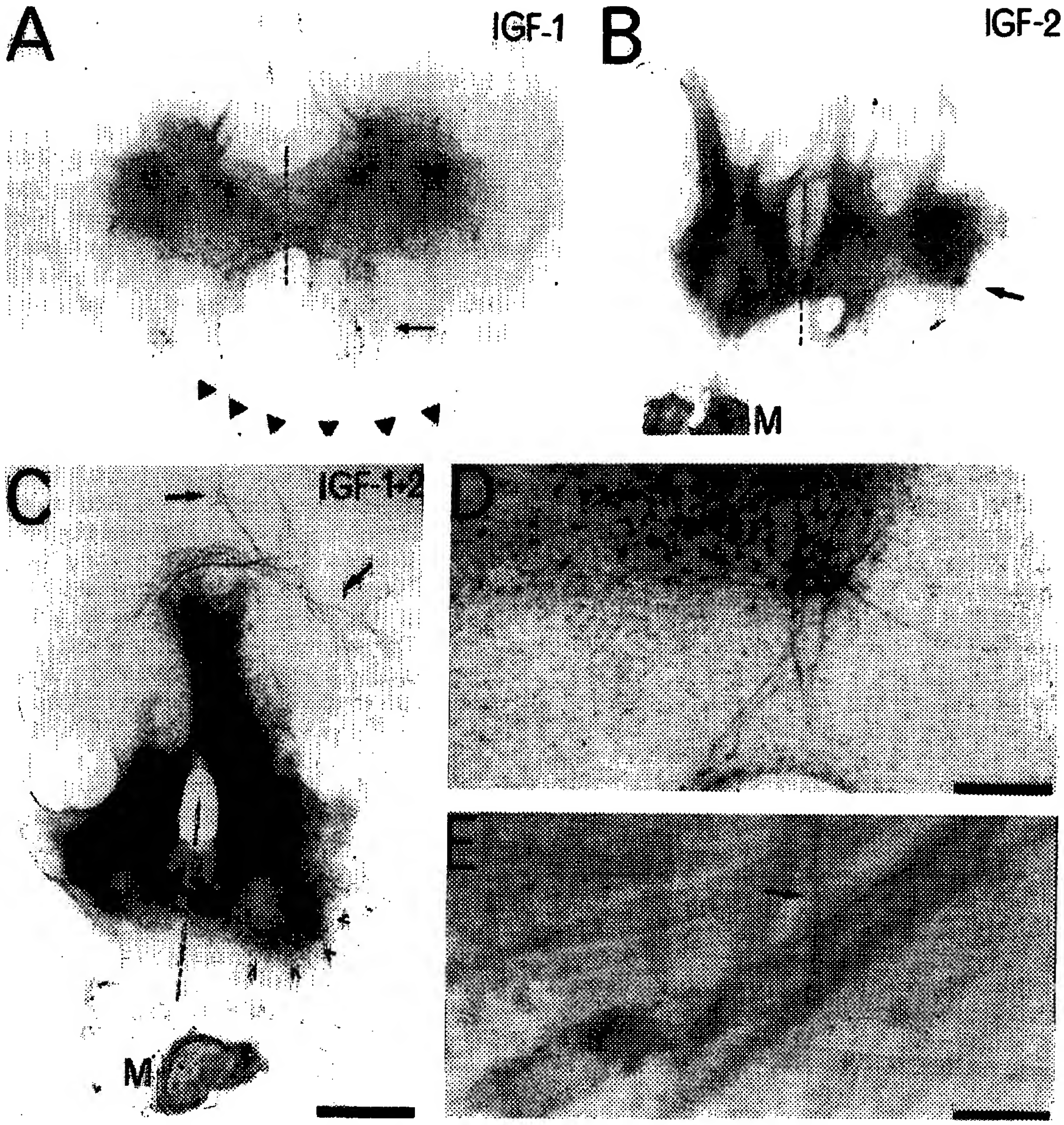


Fig. 4. Effects of IGFs added to the culture medium. Cultures at 13 DIV, stained for AChE with Cresyl violet counterstaining. Dotted lines show dorso-ventral mid-line. M, muscle tissue. A: effect of IGF-1 on a culture from the facial region. Arrow heads indicate muscular tissue. Arrow points to migrating motoneurones. Notice dorsal spread of the vestibular nuclei. B: effect of IGF-2 on a culture from the Hypoglossal region. C: effect of IGF-1 and IGF-2 on a culture from the hypoglossal region. Notice newly formed muscle tissue, migrated hypoglossal nucleus (arrow heads) and extensive arborisation of dorsal neurites (arrows). D: high power photomicrograph of the group of motoneurones indicated by arrow in B. E: high power photomicrograph of the group of newly formed muscle fibres in an IGF-1 culture. Notice multi-innervated muscle fibre, AChE-positive motor end plates and afferent axon (arrow). Bars in A–C = 1 mm; D, E = 20 $\mu$m.

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Fig. 3. Effects of 5% crude foetal liver extract in the culture medium. Cultures stained for AChE with Cresyl violet counterstaining. Dotted lines show dorso-ventral mid-line. M, muscle tissue. A: Low power photomicrograph of a culture of a brainstem slice from the hypoglossal region, with a lumbar muscle explant, 8 DIV. Arrow heads delineate the tissular outgrowth. Arrow points to AChE-positive expending neurites arising from the dorsal structures. B: same type of culture as in A, after 20 DIV. Amb, nucleus ambiguus; DNV, dorsal nucleus of the vagus nerve; Hyp, hypoglossal nucleus. C: details of the dorsal part of the culture shown in B. Notice neurites originating from left DNV and neurones from the solitary tract nucleus spreading out of the brainstem. D: higher magnification photomicrograph of a group of AChE-positive sensory neurones indicated by arrow in C. E: photomicrograph of a group of hypoglossal motoneurones indicated by arrow in B. The soma (arrow head) and dendritic branching (arrow) of a migrated motoneurone are illustrated with high power phase contrast photomicrographs in F and G, respectively. Notice numerous somatic and dendritic spines and axones running around the soma and along both branches of the dendrite. H: details of regenerating muscular tissue in the culture illustrated in A (8 DIV). Bars in A, B, H = 1 mm; in C = 500 $\mu$m; in D, E = 100 $\mu$m; in F, G = 10 $\mu$m.

pools and migration of motoneurones to the monolayer region (Fig. 4A,B). IGF-1 affected also the development of muscle tissue. The original muscle fibres degenerated and newly formed myotubes were seen spreading over the entire ventral region of the brainstem (Fig. 4A). This new muscle tissue was always connected to the brainstem explant. In the muscle tissue, AChE positive muscle fibres and multi-innervation were frequently observed (Fig. 4E).

Although IGF-1 and IGF-2 had similar effects on brainstem tissue, IGF-2 induced a more complete neuronal differentiation. Both motor and sensory regions such as vestibular nuclei or nucleus of solitary tract displayed cells with long, well differentiated neurites. The culture from the hypoglossal region nucleus illustrated in Fig. 4B shows an example of pronounced glial outgrowth and an extensive dorsal spread of AChE-positive nerve cells and neurites. Differentiated motoneurones are seen on the ventral part of the culture (Fig. 4B,D). Effects on muscle differentiation were not as marked as with IGF-1. In particular, AChE positivity of muscle fibres and motor end plates were rare.

When both IGF-1 and IGF-2 were added to the culture medium, neuronal and muscular differentiation was observed. This led in particular to an important development of sensory neurites (Fig. 4C) and well differentiated motoneurone pools.

## 4. Discussion

The results described in this paper demonstrate that a diffusible substance present in E18 tongue muscle or in liver explants induces significant trophic effects on organotypic brainstem–muscle cocultures. These effects can be reproduced by addition of IGFs into the culture medium. Since the liver is one of the major sources of IGFs, and IGFs are also present at high levels in foetal and postnatal muscles [7,28] and with amounts as high in the foetal tongue as in the liver [3], our data suggests that IGFs may promote the differentiation of functional motor connections.

Due to the structural complexity and cellular diversity of organotypic cultures, a precise description and quantification of the trophic effects of IGFs on specific cellular types cannot be achieved with the precision obtained in primary dissociated cell cultures [4,12,33, 50,51]. Nevertheless, this preparation may offer an important insight into the complex trophic processes resulting from the interactions of several cellular types such as nerve cells, glial cells and muscle fibres.

Our data shows that IGFs increase the cellular outgrowth around the brainstem and muscle explant. IGFs have a potent mitogenic effect on glial cells [32,34,36] and are produced by glial cells [26,49]. IGF-1 is a potent inducer of oligodendrocyte development [10,34,35,36,37] and induces myelination [10,26,36,37].

At the muscular level, several studies have shown that IGF-1 plays a key role in muscle growth [15,29,47, 52,56]. IGFs are produced by satellite cells in regenerating muscles [29] and by skeletal muscles [7]. An autocrine secretion of IGFs induces differentiation of myoblasts [18,55,56]. IGF-1 has been shown to stimulate terminal myogenic differentiation of L6 myoblasts by inducing expression of the myogenin gene [16,17].

We also noticed a pronounced IGF-induced morphological differentiation of neurones in motor and sensory nuclei of the brainstem. Motoneurones tended to migrate out of the brainstem and sensory neurones developed long neurites extending out of the brainstem explant into the plasma clot. Neurones displayed numerous somatic and dendritic spines and muscular contractions appeared earlier. With IGF-1, an increased number of motor end plates was observed.

These observations are relevant with the fact that IGFs have pronounced effects on nerve cells, both in vivo and in vitro. They are present in the brain and their mRNA expression are highest in embryos and largely declining after birth [5,48,50,55,56]. Neuronal and glial cells display high affinity receptors for IGF-1 [8,53] and IGF-1 is a trophic factor for both, inducing RNA and DNA synthesis [33,40]. IGF-2 was demonstrated to support survival and neurite outgrowth of sensory sympathetic neurones [46] and increases speed of regeneration of sensory sciatic axons of rats [23]. IGFs exert significant growth supporting effects on regenerating peripheral nerves [26,38] and induce neurite outgrowth from chick motoneurones in vitro [9] although this may be due to some interaction with astrocytes [2]. Finally, IGFs may induce nerve sprouting in partially denervated muscles [9] and may play an important role in the development and turnover of neuromuscular synapses [28]. It was recently shown that IGFs applied in-ovo on chick embryos rescue a significant number of lumbar motoneurones [39].

It is thus probable that, in our preparation, the IGF-rich muscle explant induces a glial outgrowth of the brainstem tissue, which is particularly marked in direction of the muscle possibly due to a gradient effect. This glial outgrowth, together with the fast differentiation of muscular tissue, bridges the gap early between the two explants. IGF diffusing from the muscle explant together with a paracrine effect of IGF produced by glial cells can promote motoneuronal survival and outmigration into the monolayer region and induce axonic regeneration leading to an early arrival of axonic growth cone in the muscle tissue. IGFs effects on regenerating motor nerves and neuromuscular synapse formation may favor early access of motoneurones to target derived neurotrophic factors [27, 31,39,41,42,43,44,45]. The establishment of functional

neuromuscular connections induces complete differentiation of myotubes [13,25,57] and target size may influence motoneuronal dendritic differentiation [11,24,54]. IGFs would thus be good candidates as muscle derived neurotrophic factors in the rat embryo.

Although there is good evidence of direct effects of IGFs on motoneurones, both in vivo and in vitro, our results on organotypic cultures stress the importance of other factors such as neuro-glial interactions, target development and functional activity that are normally present in vivo and lacking in primary cultures.


## Acknowledgements

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